Cardiac nonmyocyte subpopulations: a secular congregation

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“The advent of user-friendly platforms places population-based, single-cell RNA-Seq within individual labs, opening up new vistas for exploration and discovery to see the extensively documented landscape of CNon-MS”

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Consider the myriad of conceptual influences that collectively comprise current (and ever evolving) perspectives on the biological properties of cardiac nonmyocyte subpopulations (CNon-MS) [1–9]. Followers of the field often align with a particular ‘belief system’ that advocates for selected primary influences and cell participants responsible for myocardial biological responses (Table 1). However, CNon-MS continue to defy simple categorization and prefer their secular lifestyle. As such, CNon-MS secularism represents not being bound to any ‘belief’ system, but rather belonging to a congregation of cells existing throughout the ages that continues to elude straightforward explanations despite decades of study.

There is untold depth in the biological properties of CNon-MS to be revealed. Indeed, provincial perspectives of a cardiac interstitial cell (CIC) population dominated by fibroblasts primarily mediating scar formation is dissolving away [6,10,11]. Increasingly, nuanced appreciation of the CIC touts a rich diversity of dynamic cell types that maintain myocardial homeostasis and participate in reparative and even regenerative processes in the wake of pathologic damage [12–14]. Operational definitions in the context of this commentary refer to the CNon-MS as all nonmyocyte cells in the myocardium, including resident interstitial cells, vasculature and hematopoietic interlopers passing through, whereas the CIC specifically refers to resident interstitial cells traditionally often collectively called ‘fibroblasts’ that includes an understudied mesenchymal stromal cell population garnering attention as mediators of injury responses [14–17]. Our relatively superficial understanding of CIC heterogeneity coupled with recent awareness of inflammatory response involvement in myocardial regeneration [6,18] underscores the importance of revisiting CIC and CNon-MS biology. Although the field of CIC investigation is decades old, the oft quoted phrase from Marcel Proust is particularly true: “*The real voyage of discovery consists not in seeking new landscapes, but in having new eyes*”. This commentary asserts that CIC participation in myocardial repair and remodeling is converging with myocardial regenerative medicine and that reconciling old and new views is essential to advance both fields.

CICs in myocardial repair & regeneration

Myocardial regeneration and healing is influenced by a plethora of factors including development, age, environmental stress, co-morbidities, genetics, inflammatory responses and pharmacologic interventions [19–21]. The perceived role of CICs in myocardial repair was primarily focused upon extracellular matrix remodeling and collagen deposition for many studies of these cells decades ago. For many years, the principal cell type within the CIC compartment was purported to be the fibroblast, a cell population simultaneously hailed as essential for myocardial homeostasis and injury response while also maligned for participation in maladaptive scar formation and myofibroblast formation [12]. Revisiting the issue of relative cell proportion with genetic tracing approaches tells a different story, with cells expressing endothelial markers comprising the most abundant nonmyocyte cell type in the heart [3,15]. Moreover, there is agreement that relative proportions of CICs are subject to change in response to inflammation

Table 1. The evolving perspective of cardiac nonmyocyte participants and influences (not in any particular order and with apologies to any left unrecognized).

Participants	Influences
Fibroblasts	Development
Fibrocytes	Aging
Myofibroblasts	Pathological damage
Fibromyocytes	Inflammation
Pericytes	Metabolism
Endothelial cells	Cytokines/chemokines
Inflammatory cells	Mechanical stress
Mesenchymal stem cells	Extracellular matrix proteins
Cardiac stem cells	Proteases
Epicardial cells	Oxidative stress
Vascular smooth muscle cells	Hypoxia

and repair following pathological injury [6]. Thus, CIC cellular composition is in a dynamic equilibrium state that changes in response to environmental cues and multifaceted stimuli including development, damage, chronic or acute stress, as well as aging.

The collision course between CICs and regenerative medicine was predictable, but the disparate nature of trajectories to link these fields together has left them somewhat disjointed and sometimes seemingly at odds with one another. Regenerative research in myocardial biology is well over a dozen years old since inception of the resident cardiac stem cell (CSC) concept [22,23]. Resident CSCs are unquestionably a subpopulation of CICs based upon localization within the interstitium and phenotypic properties such as morphology, proliferation in response to injury, secretion of paracrine factors involved in remodeling and plasticity [24]. Ample evidence supports the premise that reparative potential of CSCs declines with age and that biological properties of this population differ between young and old hearts, likely accounting for impaired capacity of the aged heart to recover from pathologic stress [22,25–28]. Association of c-kit marker expression with CICs of mesenchymal and fibroblast lineage is well established [29–31]. Several studies tout ability of fibroblasts to be reprogrammed into the cardiomyocyte lineage by inducible pluripotent stem cell technology [32–35]. The blurring of distinctions between CIC and CSC is becoming increasingly clear and highlights two fundamental issues plaguing advancement of myocardial biology: lack of clarity regarding biological relationships of CICs to each other and rudimentary understanding of CIC population heterogeneity.

Advances in transcriptional profiling for heterogeneity characterization

Heterogeneity is a well-recognized but poorly defined characteristic of the CIC population [5,11,36]. CIC population census-taking is made more challenging by aforementioned dynamics in response to environmental changes as well as subtle spectrum diversity of differences that manifest as superficially indistinguishable cells. Inevitably, approaches to characterize cellular heterogeneity are inherently limited by experimental design that may introduce selectivity bias (intentional or otherwise) through use of antibody markers, adherence, cell culture or selective depletion to choose target populations. Concomitantly, oversimplification of heterogeneity results from pooling of cell types together under arbitrary criteria that are unavoidable with bulk population characterizations. Transcriptional profiling is increasingly popular for characterization of cellular signatures allowing quantitative assessment of transcript levels representing thousands of distinct genes within a cell population or even individual cells. Prior studies of CNon-MS or CICs using transcriptional profiling revealed substantial insights regarding cellular diversity, but often fall short of assessing either individual cell properties or capturing population heterogeneity. A few recent examples will suffice for illustration of the many studies profiling CNon-MS or CIC transcriptome. One such analysis of multiple cardiac cell populations identified several regulated gene sets exhibiting differential transcriptional activity in response to developmental or infarction injury cues, but experimental design began with flow cytometry separation of CNon-MS based upon several antibody markers, each possessing selectivity for limited cell subpopulations [37]. Another recent report touting ability of c-kit⁺ cells to differentiate into cardiomyocytes by the van Berlo lab used single-cell RNA-Seq (scRNA-Seq) analysis on the Fluidigm platform, sequencing of an enriched CD117⁺/CD45[−] population from 88 neonates, 171 C56/BL6 adults, and 146 c-kit knock-in mice [38]. Importantly, the van Berlo

publication highlights c-kit-enriched population heterogeneity and acknowledges potential for overlooking rare cells due to small sampling size that "... *may have missed identification of this small (cardiogenic) population of c-kit⁺ cells* ...". Another unrelated study relied upon genetic lineage tracing to define fibroblast (Tcf21⁺) or myofibroblast (periostin⁺) cell populations by promoter-driven fluorophore expression that approached transcriptome changes using scRNA-Seq [3]. This characterization relied upon flow cytometry negative selection against CICs of endothelial (CD31) or myeloid (CD45) lineage with the rationale that such cells 'contaminate the fibroblast single-cell analysis.' However, arrival of CD45⁺ 'fibrocytes' as a CIC in the scar area following infarction or ischemia/reperfusion injury is well known [39]. A third exciting study breaks down longstanding barriers between CIC and CSC through transcriptional profiling, but characterized short-term cultured CIC using Agilent microarray analyses on bulk RNA preparations [10]. This same group created 'CARFMAP: a curated pathway map of cardiac fibroblasts' using transcriptome data derived from the collective literature of approximately 180 PubMed articles and over 30 data series specifically investigating cardiac fibroblasts [40]. Resulting analyses reveal differentially expressed genes are limited between fibroblasts of cardiac versus tail to 3.1% of the transcriptome, with five of the identified genes strongly associated with cardiovascular and other critical cell functions (*Mmp3*, *Il6*, *Edn1*, *Pdgfr* and *Fgf10*). A supportive precedent recently demonstrated scRNA-Seq analysis of the nonmyocyte cardiac cellular landscape confirming transcriptomic diversity and potential networks of intercellular utilizing the 10× Genomics Chromium platform [41]. Notably, this descriptive study by Skelly *et al.* [41] used normal healthy C57BL/6J (two male, two female) mice for freshly isolated nonmyocyte preparations depleted for endothelial cells by flow cytometry prior to transcriptomic analyses with the rationale that endothelial cells represent the largest cardiac cell population [15]. Such preemptive biasing of the nonmyocyte population skews results and, importantly, nonbiased transcriptomic profiling of myocardium demonstrates fibroblasts (not endothelial cells) represent the predominant cell type [42], in agreement with our findings using cardiac interstitial cell preparations [43]. Thus, fundamental differences in experimental design approach between Skelly *et al.* [41] versus other recent scRNA-Seq studies [42,43] demonstrate the critical need for additional unbiased and rigorously designed transcriptomic profiling analyses. All these studies explore CNon-MS and CIC population heterogeneity and suggest candidate marker genes, yet selection for optimal biological properties mediating myocardial repair and regeneration is rarely pursued.

Transcriptional profiling has historically depended upon fractionation of cell populations to narrow target cell focus using selective markers (antibody surface protein expression) or phenotypic properties (dye exclusion, adherence, size). Complex biological systems such as the myocardium and the CNon-MS possess spectrum heterogeneity across multiple distinct cell populations that can only be revealed by single-cell level analyses. Implementation of scRNA-Seq has been used to infer cell lineages, identify subpopulations and highlight cell-specific biological characteristics [44–49]. Future advancements will enable single-cell multiomics to correlate aspects of genome, epigenome, transcriptome and proteome profiling [50]. While recent scRNA-Seq advances have been revolutionary, researchers must also be mindful how to best implement this exciting technology to avoid pitfalls and limitations inherent in this exciting new approach [51–59]. Massively parallel single-cell transcriptomics has arrived as a platform for cell/molecular biology laboratories [44] to delve into scRNA-Seq of the CNon-MS, CIC and CSC.

Recently, our group published scRNA-Seq studies of freshly isolated CNon-MS populations to understand their inherent biological properties and heterogeneity. Beginning with comparison of fresh isolates to cultured cells to define adaptations and alterations resulting from natural selection pressure *in vitro*, we reported that *ex vivo* expansion of CSCs promotes increased transcriptome diversity despite loss of tissue-specific marker identity. Interestingly, transcriptome alterations prompted by *ex vivo* expansion increase population homogeneity compared with freshly isolated CPCs extracted from their native niche *in vivo* microenvironment. Smart-seq2 datasets confirmed the suggestion that cultured cells exhibit greater transcriptome similarity than freshly isolated cells. In summary, environmental influence of cell culture promotes transcriptome migration toward a common shared profile throughout the population with minimal unique signatures [43].

Conclusion

Analyses using freshly isolated CNon-MS have fundamental importance for understanding myocardial biology in development, injury, and aging, as well as effects of cell culture with implications for cell therapy and regenerative medicine. The proverbial 'tip of the iceberg' is being examined in terms of cellular relationships that can be mined from massively parallel scRNA-Seq. CICs remains one of the great frontiers in myocardial biology [11,12,18] as technical capabilities to recognize complexity and plasticity continues to mature. The advent of user-friendly platforms

places population-based, single-cell RNA-Seq within individual labs, opening up new vistas for exploration and discovery to see the extensively documented landscape of CNon-MS [16,39,40,60] with new eyes.

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